

Multiplexed SELEX for Sulfonamide Antibiotics Yielding a Group-specific DNA Aptamer for Biosensors

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Abstract

The widespread use of sulfonamide (SAs) antibiotics in animal husbandry has led to residues of SAs in the environment, causing adverse effects to the ecosystem and risk of bacterial resistance, which is a potential threat to the public health. Therefore, it is highly desirable to develop simple and high-throughput methods that can detect multiple SAs simultaneously. In this study, we isolated three kinds of aptamers with different specificities based on a multi-SA systematic evolution of ligands by exponential enrichment (SELEX) strategy using a mixture of sulfadimethoxine (SDM), sulfaquinoxaline (SQX), and sulfamethoxazole (SMZ). Three aptamers were obtained and one of them showed a similar binding to all tested SAs, with dissociation constant (K_d) ranging from 0.219 μM to 0.63 μM . For the other two aptamers, one is specific for SQX, and the other is specific for SDM and sulfaclozine. A label-free detection method based on the broad-specificity aptamer was developed for the simultaneous detection of six SAs, with detection of limits ranging from 0.14 μM to 0.71 μM in a lake water sample. The aptasensor has no binding for other broad-spectrum antibiotics such as β -lactam antibiotics, quinolones, tetracyclines, and chloramphenicol. This work provides a promising biosensor for rapid, multi-residue and high-throughput detection of SAs, as well as a shortcut for the preparation of different specific recognition elements required for the detection of broad-spectrum antibiotics.

Keywords: Sulfonamides, aptamers, multiplexed SELEX, biosensor, label-free

Introduction

Sulfonamides (SAs) are low-cost, broad spectrum synthetic antibiotics. They are widely used to treat systemic bacterial infections.¹ More than 40 kinds of SAs have been used in human and veterinary medicine, among which sulfadiazine (SD), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfamethoxazole (SMZ), sulfaclozine (SCZ), and sulfathiazole (STZ), are most common.² The irrational use or abuse of SAs in animal husbandry can lead to SA residues in the environment (e.g., water), causing adverse effects to the ecosystem and bacterial resistance.³ Therefore, there is a great need to detect them.

Current analytical methods for SAs include the use of liquid chromatography (LC)⁴ and LC-tandem mass spectroscopy (LC-MS/MS).^{5,6} The above instrument methods can simultaneously measure multiple SA antibiotics, but they require expensive instruments, professional operators, high cost and a long turnaround time. So, they are not suitable for on-site rapid monitoring of SAs. Immunoassay such as ELISA⁷ and immunochromatography⁸ are simple to use, fast and cost-effective suitable for field testing. However, antibodies are mainly prepared by animal immunization. The success rate of antibody production depends largely on the design and experience of researchers and the animal, leading to large batch-to-batch variation.⁹ Therefore, immunoassays are also susceptible to poor reproducibility.

For the detection of small molecules, aptamers have many advantages.^{10,11} Since 2012, many aptamers have been reported for the detection of SAs. For example, SDM,¹² sulfanilamide (SNL),¹³ and sulfameter (SM)¹⁴ were immobilized on magnetic beads for aptamer selections. Target immobilization may block part of target molecules also leading to suboptimal aptamers. Le's group used GO-SELEX to isolate aptamers for SQX,¹⁵ SD,¹⁶ and sulfamethazine (SMT).¹⁷ Recently, He et al used capture-SELEX to immobilize a DNA library via hybridization to select aptamers for SM.¹⁸ The large amount of work on SAs aptamers indicate the widespread interest for their detection. However, we have identified a few critical research gaps. First, all the selected aptamers showed binding only to the target sulfonamide. While this may demonstrate specificity of aptamers, a general aptamer that can bind this group of antibiotics might be more useful, since it is unlikely to develop over 40 different aptamers to detect them individually. Second, most of the previous work only used a single SA as target, and using a mixture of a few similar SAs might help understand

aptamer evolution and identification of aptamers that can bind to SAs as a group. Finally, all the previous work used nanomaterials as part of aptamer binding assay. In addition to using GO, many other types of materials were also employed including gold nanoparticles (AuNPs). We demonstrated that AuNPs can result in artifacts in aptamer binding assays.¹⁹⁻²¹ Therefore, it is important to validate aptamer binding using more rigorous label-free assays.²²⁻²⁴

In this work, we used a mixture of SDM, SQX, and SMZ as targets for capture-SELEX of aptamers. Interestingly, we discovered aptamers that can bind to them as a group. In addition, specific aptamers that can only recognize one or a few of SAs were also obtained. Label-free fluorescence biosensors were developed using these aptamers.

Materials and Methods

Chemicals

All of the DNA samples were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and their sequences are listed in Table S1. Streptavidin-coated agarose resin (1-3 mg biotinylated BSA/mL loading capacity) was bought from Thermo Scientific (IL, USA). SDM, SQX, SMZ, SCZ, STZ, trimethoprim (TMP), thioflavin T (ThT), and SYBR Green I (SGI) were from Sigma-Aldrich. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NaCl, MgCl₂, and NaOH were obtained from Bio Basic (Toronto, ON, Canada). 3 k and 10 k Ultra-0.5 centrifugal filter unit were from Millipore-Sigma. Micro bio-spin chromatography columns and SsoFast EvaGreen supermix were from Bio-Rad. dNTP mix, Taq DNA polymerase with ThermoPol buffer, and the low molecular-weight DNA ladder were from New England Biolabs (Ipswich, MA).

SELEX

The SELEX experiments were performed according to previously described procedures.^{10, 25} The selection buffer contained 20 mM HEPES, pH 7.4, 100 mM NaCl and 10 mM MgCl₂. The SDM, SQX, and SMZ stock solutions were prepared in the selection buffer at a concentration of 10 mM. The concentration of the three SAs from round one to five was 1 mM each, from round six to twelve 0.2 mM each, and from

round thirteen to fifteen 0.05 mM each. The PCR products of round 12 (a mixed sample) and round 15 (three separately eluted samples) were sent for sequencing.

ThT fluorescence assays

ThT-based fluorescence assays were performed on a Variant Eclipse fluorescence spectrophotometer. 2.5 μ L aptamer (100 μ M), 5 μ L ThT (100 μ M), and 5 μ L Triton X-100 (1%) were added in 487.5 μ L of buffer (20 mM MES, pH 5.8, 50 mM NaCl, 10 mM MgCl_2). The solution was transferred to a quartz cuvette and a SA drug was gradually titrated to a final concentration of 15 μ M. The excitation wavelength was set at 440 nm, and the emission was monitored from 450 nm to 530 nm. The fluorescence values at 490 nm were used for calculation. The dissociation constant (K_d) was fitted using the following equation: $y = y_0 + aK_d/(K_d + x)$, where x is the concentration of the titrated target, and a is the maximal fluorescence change upon saturated binding.

SFI fluorescence assays

The process of SFI assay was performed following the same procedure as the above ThT assay, except that the excitation wavelength was set at 480 nm, and the emission wavelength was monitored from 490 nm to 560 nm. The fluorescence values at 520 nm were used for calculations. The final SFI concentration was 1 μ M. The lake water was collected from Lake Ontario. The assay was performed using the SDM-3 aptamer and SFI as described above. After the lake water was filtered, different concentrations of SAs were spiked, and each concentration was tested in triplicate.

Isothermal titration calorimetry (ITC)

ITC was performed using a MicroCal VP-ITC. First, 2 mL ITC buffer (20 mM MES, pH 5.8, 50 mM NaCl, 10 mM MgCl_2 , 1% ethanol for MIX-1; 20 mM HEPES, pH 7.0, 20 mM MgCl_2 , 1% ethanol for SDM-3) was prepared. Then, an aptamer (10 μ M) in the buffer was annealed by heating to boiling for 5 min and cooling naturally over 30 min. Second, 200 μ M target was also dissolved in 2 mL ITC buffer. The above aptamer and target solutions were degassed for 5 min prior to loading. Then 281 μ L of target solution was filled in the syringe and 1.45 mL of aptamer solution was injected into the cell chamber. Except for an

initial injection of 0.5 μ L, 10 μ L of the target was titrated into the cell each time over 20 s duration for a total of 28 injections at 25°C. The titration spacing was set for 360 s between each injection. The K_d value was obtained by fitting the data to a one-site binding model using the accompanying Origin software.²⁶

Results and Discussion

Aptamer selection

Our DNA library contained a 30-nucleotide (nt) random region flanked by two constant primer-binding regions. The library was immobilized by hybridization to an oligonucleotide bearing a biotin tag attached to streptavidin-functionalized agarose beads.²⁷⁻³¹ The structures of the three SA targets are shown in Figure 1A, and their mixture was used for the selection. In addition, we also included three other SAs for selectivity assays (Figure 1B). DNA sequences that can bind to one of the three target SAs were released from the beads and collected. The initial five rounds of selection were performed with 1 mM each of the SAs. For the next 7 rounds, the SA concentration was dropped to 0.2 mM each, and to 0.05 mM each for the last 3 rounds. After the final round, we split the library into three and eluted them separately with a different target. We sequenced all the three separated libraries along with the mixed library at the end of round 12 (before the further decrease of target concentration).

All the sequencing results were highly converged, suggesting successful enrichment of aptamers. The top 6 sequences from each of the sequenced library is shown in Table S2. Out of all them, we identified seven sequences that seem to be different from each other (Figure 1C). We color coded the identical nucleotides and found that many motifs are present in different sequences. These aptamers were named as follows. MIX-1 means the most abundant sequence in the mixed library, and SDM-3 means the third most abundant sequence from the sulfadimethoxine eluted library, for example.

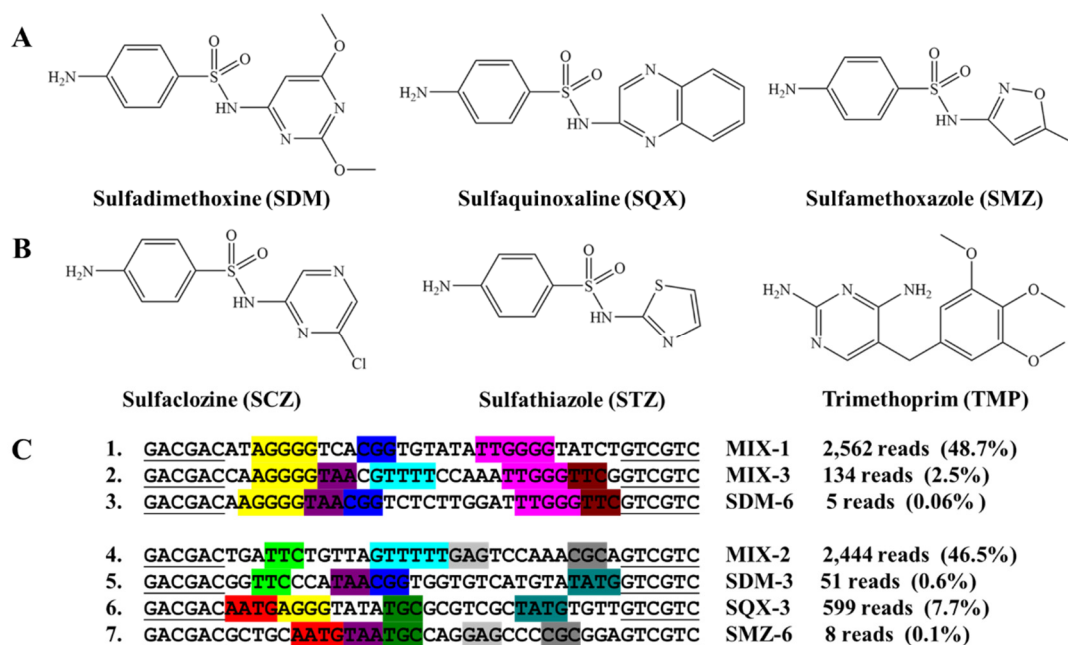


Figure 1. The structures of (A) SDM, SQX, and SMZ, and (B) SCZ, STZ, and TMP. (C) The seven unique sequences from the SA selection. Their copy number and percentage in their respective library are also shown. The color highlighted regions represent conserved nucleotides found in different sequences.

Of these seven sequences, the first three contained many common regions, and we classified them as the same family. Thus, only five sequences had obvious differences, and we picked them for binding assays. The predicted secondary structures of the five tested sequences are shown in Figure 2.³² Two of them had a simple hairpin structure, whereas the rest three have internal stem loops.

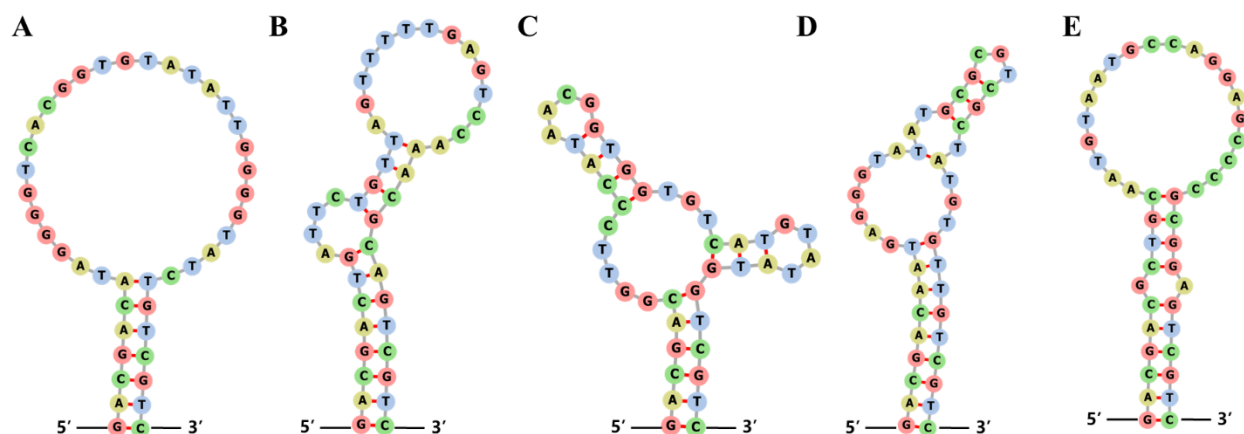


Figure 2. The predicted secondary structures of five aptamers: (A) MIX-1, (B) MIX-2, (C) SDM-3, (D) SQX-3, and (E) SMZ-6. The structures were generated using RNAalifold.

ThT-based binding assays

Some of the aptamers (e.g. MIX-1) are rich in guanine in the loops, suggesting that thioflavin T (ThT) might be a useful fluorescence probe for label-free binding assays.³³ Thus, we first used ThT fluorescence spectroscopy to evaluate binding of the aptamers. When ThT binds to an aptamer, its fluorescence was enhanced. When a target molecule binds to the aptamer, ThT might be displaced resulting in a decreased fluorescence. Using the MIX-1 aptamer as an example, we observed a pronounced decrease in fluorescence in the presence of SDM (>65%), but a much smaller change in fluorescence with SMZ or SQX (<20%) (Figure 3A). Similarly, for MIX-2 and SMZ-6, the same was observed (Figure 3B and 3C). From this selectivity pattern, these three aptamers might have a similar way of binding. Since these aptamers represent the majority of the mixed library, it is likely that even at round 12, the library was dominated by SDM binding sequences. It implies that SDM is a more favorable target for enriching aptamers. The other two sequences (SDM-3 and SQX-3), however, did not show any response to the SAs with ThT (Figure S1).

Aside from the three target molecules, we tested three more SAs, including sulfaclozine (SCZ), sulfathiazole (STZ), and trimethoprim (TMP). Interestingly, SCZ showed even better binding, although the other two SAs showed no binding to these three aptamers. It is understandable that removing the methoxyl groups can better fit the drug in the binding pocket since methoxyl groups themselves do not have a strong interaction mechanism with aptamers. The Stojanovic group recently dissected the contribution of different functional groups to aptamer binding.³⁴ Although the authors did not specifically examine methoxyl groups, our result can be rationalized based on this concept. The difference in K_d between these three aptamers for SCZ and SDM was quite small, suggesting that the majority of binding was from the common sulfonamide part. Yet, the structure of the other part is also critical, since none of the rest SAs can bind. So far, we discovered a few new aptamers that have the best selectivity for SCZ followed by SDM.

SYBR Green I based binding assay

Since SDM-3 and SQX-3 did not respond to ThT, we then used SYBR Green I (SGI) to monitor their binding. SGI prefers to bind to duplex regions and it may complement to ThT for probing aptamer binding.

Very interestingly, for the SQX-3 aptamer, we observed ~50% fluorescence increase with SQX, while no fluorescence change occurred for the other SAs (<5%, Figure 3D). We noticed that the abundance of this sequence was high (7.7%), and compared with the other two isolated libraries, the enrichment of SQX-3 was significantly higher than that of SMZ and SDM (Table S2). This result may explain the survival of SQX-3.

For the SDM-3 aptamer, it showed fluorescence decreased in the presence of all the 6 SAs, and the fittings showed that they all had K_d values below 1 μ M. Given the difference in these six drugs, this aptamer mainly binds to the common region of the SAs, and is a broad-spectrum aptamer for SAs (Figure 3E). Therefore, based on the SGI binding assay, two new aptamers were discovered: one specifically binding to SQX, and the other binding SAs as a group. Table S3 summarizes the determined affinities of the selected aptamers by using the ThT-based and SGI-based assays.

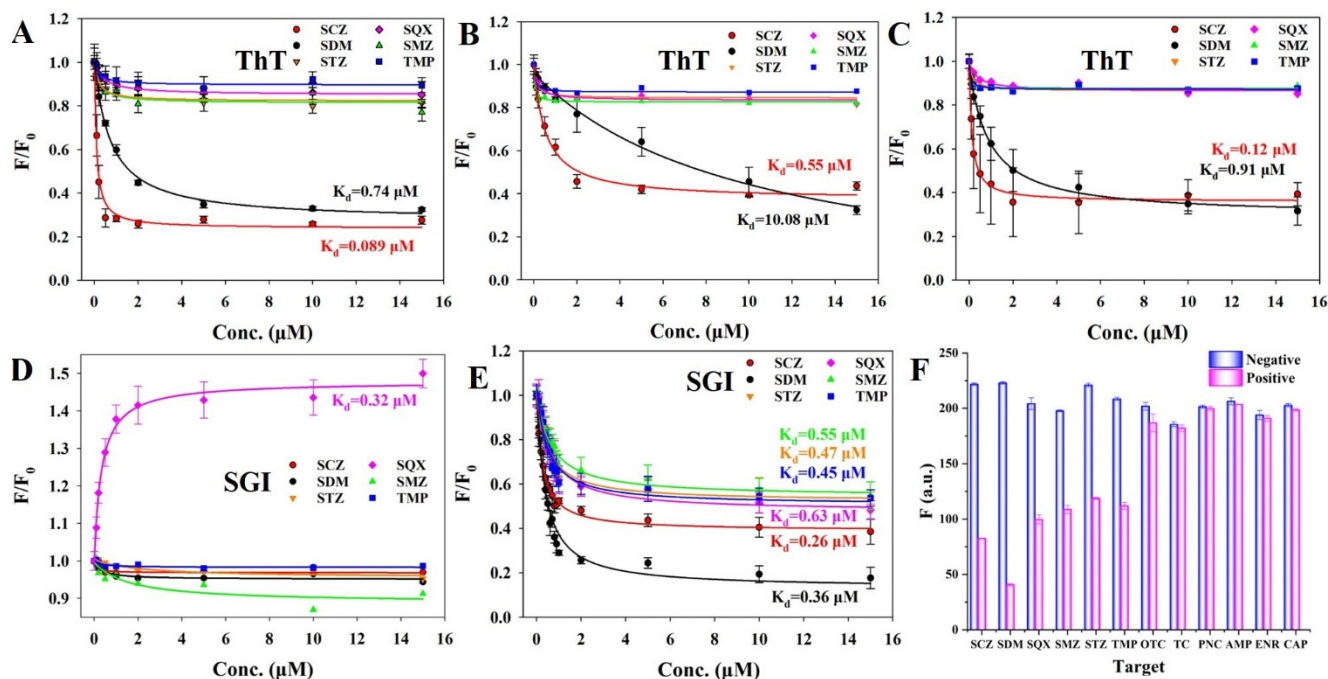


Figure 3. The ThT-based binding assays of (A) MIX-1, (B) MIX-2, and (C) SMZ-6 to the six SAs. The SGI-based binding assays of (D) SQX-3 and (E) SDM-3 to the six SAs. (F) Selectivity of the SDM-3 aptamer to different drugs. Negative means samples without a target, and positive means with 1 μ M SAs drugs or 200 μ M other targets. OTC: oxytetracycline; TC: tetracycline, AMP: ampicillin; PNC: penicillin, ENR: enrofloxacin; and CAP: chloramphenicol.

Implication of different responses to ThT and SGI

It is interesting that two aptamers only responded to SGI while the rest three can be signaled using ThT. Therefore, for an aptamer, it is important to screen for different dye probes. The lack of a dye response does not necessarily mean that there is no binding. The Bhaskar team³⁵ studied nucleic-acid-binding dyes as indicators of aptamer-target interactions for the ATP, thrombin, and theophylline aptamers. They used five different dyes (YOYO, ethidium bromide, thiazole orange, SYBR Green I, and Hoechst 33258) for binding assays. The authors concluded that the success of a signaling strategy not only depends on the degree of aptamer conformational change upon target binding but also on the property of the dye used. Our observations here are consistent with their results.

ITC

ITC was used to further confirm the affinity of aptamers to SAs. ITC is a label-free homogeneous method to obtain all the thermodynamic parameters including K_d .³⁶ The lower the K_d , the stronger the affinity of an aptamer.³⁷ In this study, 200 μ M SCZ or SDM was titrated into 10 μ M aptamer and an exothermic reaction was observed. Because MIX-1, MIX-2, and SMZ-6 aptamers all have the best selectivity with SCZ, we tested MIX-1 using ITC. The fitted K_d value was 118 nM (Figure 4A), which was similar to that from the ThT assay (89 nM). The K_d for the SDM-3 aptamer for SCZ and SDM was 219 nM and 247 nM using ITC, respectively (Figure 4B and 4C), and the K_d values were 260 nM and 360 nM by SGI binding assay. Therefore, in this case, these two techniques gave essentially the same result for these aptamers. In general, ITC is a more reliable measurement of aptamer affinity, since there are no other components that interfere binding.³⁸ This result verifies binding of the aptamers and reliability of the dye probes. The thermodynamic values of the aptamer binding reactions are shown in Table 1, and all the binding reactions were enthalpy driven.

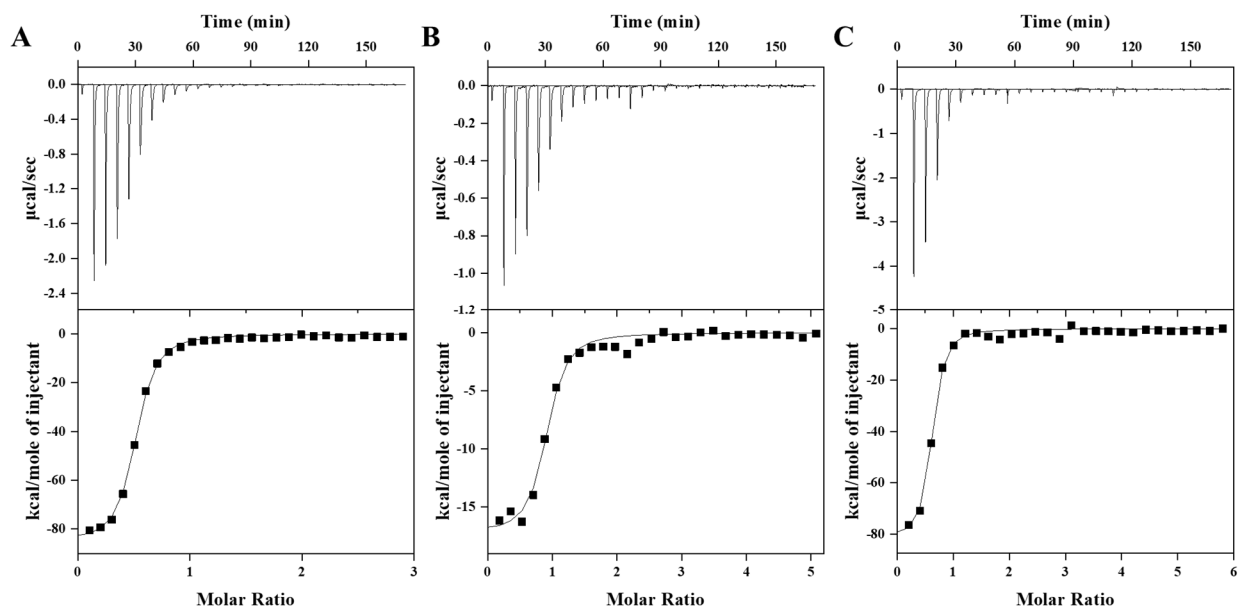


Figure 4. ITC results of aptamer binding by titrating 200 μM SCZ into (A) 10 μM MIX-1, and (B) SDM-3 aptamers, and (C) 200 μM SDM into 10 μM SDM-3 aptamers.

Table 1 The thermodynamic values of the binding.

Aptamer	N	Target	ΔH (kcal/mol)	ΔS (cal/K·mol)	K_d (nM)
MIX-1	0.59 ± 0.01	SCZ	-85.4 ± 0.9	-255	118
SDM-3	0.86 ± 0.02	SCZ	-17.3 ± 0.5	-27.5	219
SDM-3	0.68 ± 0.009	SDM	-82.2 ± 1.9	-244	247

N : binding stoichiometry

Optimization of detection conditions

Among the aptamers we obtained in this study, SDM-3 is the most interesting since it can bind to all the six SA antibiotics, which was not achieved previously. Therefore, the rest of the work was focused on this aptamer. There are many factors that affect aptamer binding,^{39, 40} such as aptamer concentration, the type and pH value of buffer, and salt concentration. To use SDM-3 as a broad-spectrum sensor for SAs, we first optimized aptamer concentration, with the ratio of aptamer to SGI fixed at 1:2. Figure 5A shows that the fluorescence changed less with increasing aptamer concentration. This is reasonable since a sensor is

more sensitive with a lower probe concentration. The fluorescence decrease was 62.8% and 62.6% when the aptamer concentration was 25 nM and 50 nM, respectively. Although the fluorescence change was similar, the fluorescence intensity at 25 nM was only one-third of that at 50 nM, and the signal error was twice that at 50 nM. So, we chose 50 nM as the optimal aptamer concentration.

The effect of pH was then investigated. As the pH was raised, the fluorescence change first increased and then decreased. Figure 5B shows that when the pH was 7.0, the fluorescence change was the largest. Therefore, pH 7.0 was the optimal pH. Similarly, Figure 5C shows that 20 mM Mg^{2+} achieved the largest signal change. Finally, we tested the effect of Na^+ concentration, and the signal change was quite independent of Na^+ . The largest change in fluorescence was achieved in the absence of Na^+ (Figure 5D). Since this aptamer requires Mg^{2+} for binding, adding Na^+ may compete with Mg^{2+} . Different aptamers have different optimal conditions. For example, the optimal pH for the MIX-1 aptamer was pH 5.8 (Figure S2).

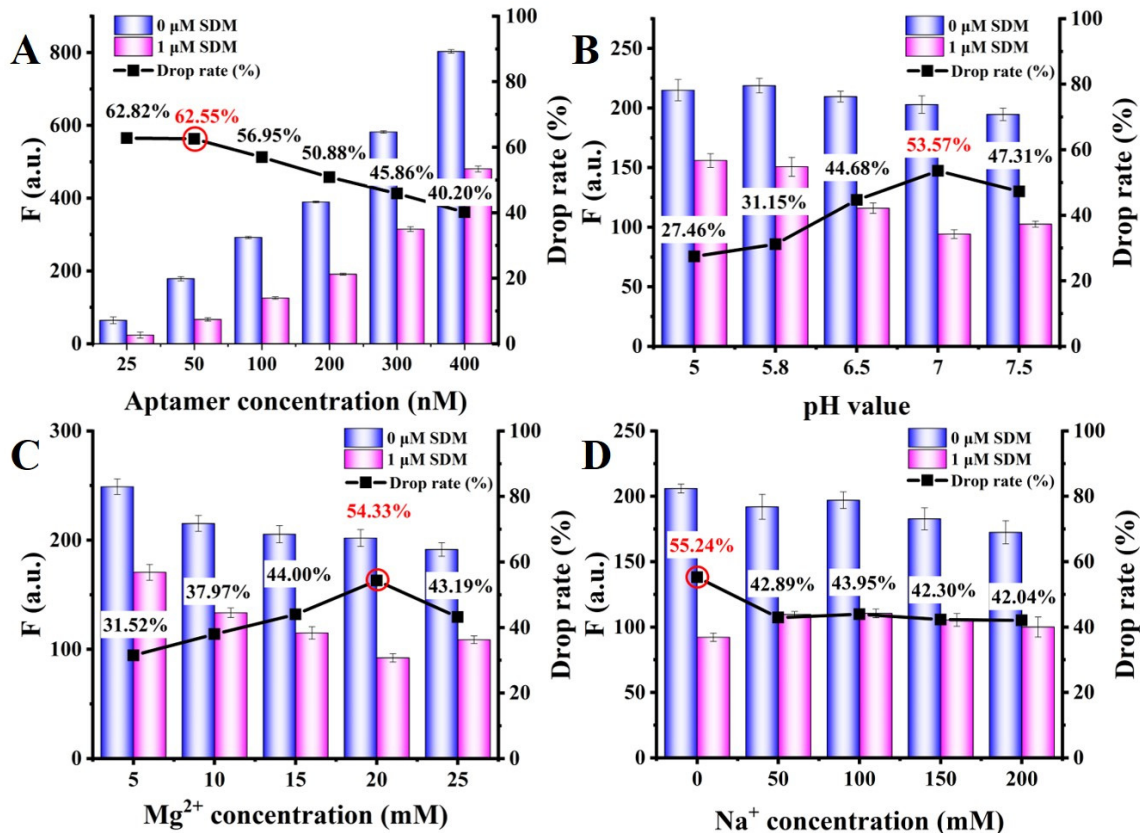


Figure 5. Effects of different factors on SDM-3 aptamer binding, including (A) aptamer concentration; (B) pH; (C) Mg^{2+} concentration; and (D) Na^+ concentration.

Sensitivity and selectivity of SDM-3 based sensor

With the above optimized condition, the sensitivity and selectivity of the SDM-3 based sensor were measured. A series of concentrations of drugs were added to the SDM-3 and SGI mixture and the fluorescence was recorded. The detection limit (LOD) was calculated based on $3\sigma/\text{slope}$, where σ is the standard deviation of the background fluorescence variation. For the SDM-3 broad-spectrum recognition aptamer, the LODs of detecting SCZ, SDM, SMZ, SQX, STZ and TMP were 0.15 μM , 0.07 μM , 0.23 μM , 0.41 μM , 0.35 μM , and 0.31 μM , respectively. Then, we evaluated the selectivity of SDM-3 to other classes of antibiotics, including oxytetracycline (OTC), tetracycline (TC), ampicillin (AMP), penicillin (PNC), enrofloxacin (ENR), and chloramphenicol (CAP). The results (Figure 3F) showed that when the concentration of these drugs was as high as 200 μM , no change in the fluorescence was observed, indicating that SDM-3 specifically binds to SAs.

Real sample tests

To verify the reliability and practicality of our sensors, we further challenged our sensor in lake water. There are many unknown components in lake water, which may cause matrix interference to the sensor. After the lake water was filtered, different concentrations of SAs were spiked, and each concentration was tested in triplicate. The results showed that the sensor can robustly achieve the specific recognition to SAs in complex matrix samples. The LODs of the biosensor for SCZ, SDM, SMZ, SQX, STZ and TMP were 0.48 μM , 0.14 μM , 0.42 μM , 0.18 μM , 0.58 μM , and 0.71 μM , respectively (Figure 6). Therefore, this aptasensor has great application potential in the field for environmental monitoring.

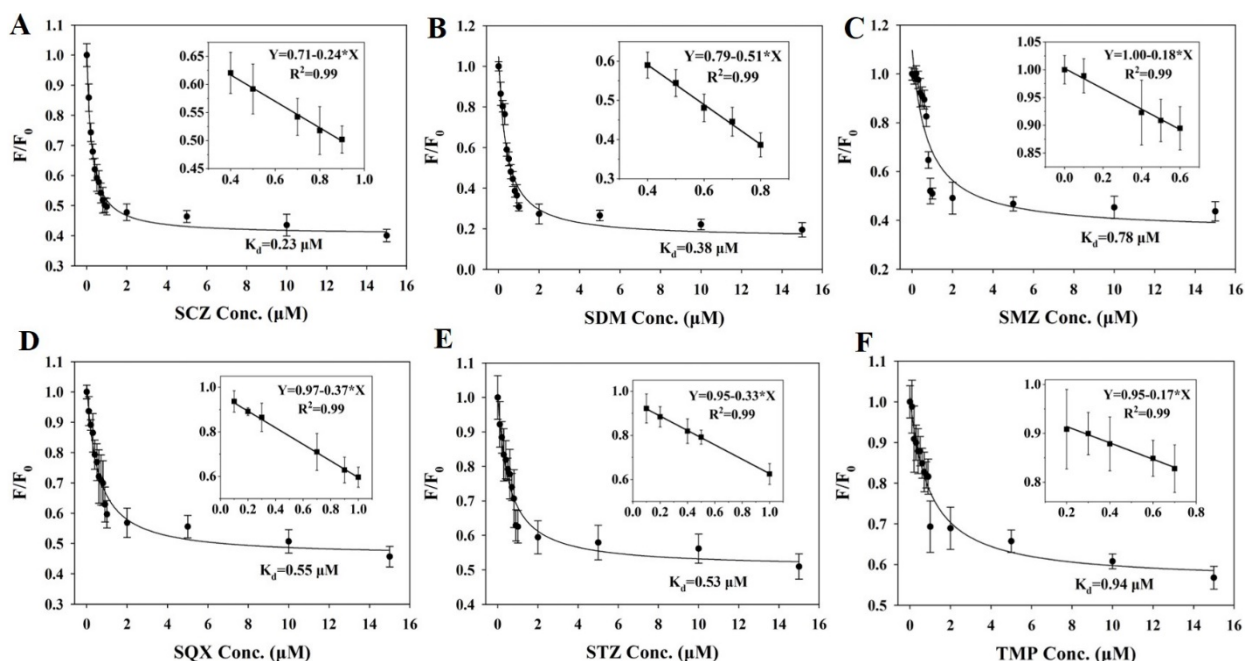


Figure 6. Detection of 6 kinds of SAs in Lake Ontario water by the SDM-3 aptasensor. (A) SCZ; (B) SDM; (C) SMZ; (D) SQX; (E) STZ; (F) TMP. The insets share the same x- and y-axis as the main figures.

Comparison with previously reported aptamers

Table 2 has summarized the previously reported SA aptamers, and the reported aptamer sequences are listed in Table S4. There are a few major differences between our aptamers that the previous ones. First, most previous works used a single SA target, and the selected aptamers showed binding only to the target SA. Only one previous study used four different SAs as targets, the four drugs are sulfamethoxypyridazine (SMPZ), sulfathiazole (STZ), sulfamerazine (SMR), and sulfapyridine (SPD).⁴¹ However, the best aptamer they obtained named Sull-4 only had good binding to SQX (K_d 670 nM), the K_d values for other drugs were all greater than 20 μ M from microscale thermophoresis. Therefore, this is not a broad-spectrum aptamer either. For practical applications, a general aptamer that can bind this group of antibiotics might be more useful. Second, we aligned the seven sequences we obtained with the literature reported aptamers (Figure S3). We could not find sequence homology between our new aptamers and any of the reported aptamers, indicating that new aptamers were obtained from our selections.

Table 2 Comparison with previously reported aptamers

Aptamer	Target for selection	Optimal length	K_d (nM)	Method for K_d	Selectivity	Ref.
SDZ30-1	SD	13 nt	65	MBs-based labeled fluorescence assay	SD	Tang et al ¹⁶
SBA2-1	SQX	38 nt	82	GO-based labeled fluorescence assay	SQX	Shi et al ¹⁵
SMZ1S	SMT	9 nt	24	GO-based labeled fluorescence assay	SMT	Kou et al ¹⁷
SA07	SMT	60 nt	79	Equilibrium filtration method	SMT	Yang et al ⁴²
Su13	SDM	22 nt	84	MBs-based labeled fluorescence assay	SDM	Song et al ¹²
Sulf-1	SM	58 nt	154	GO-based labeled fluorescence assay	SM	He et al ¹⁸
SME4-1	SM	13 nt	84	MBs-based labeled fluorescence assay	SM	Yan et al ¹⁴
Sull-04	SMPZ, STZ, SMR, and SPD	51 nt	670-109000	BHQ-cDNA labeled fluorescence assay	SQX (K_d 0.67 μ M). SMPZ, SMD, SCP, and SPD (K_d > 20 μ M)	Xu et al ⁴¹
SDM-3	SDM, SQX, and SMZ	42 nt	219-630	ITC and SGI-based label-free fluorescence assay	SDM, SQX, SMZ, SCZ, STZ and TMP (K_d < 1 μ M)	This work
MIX-1	SDM, SQX, and SMZ	42 nt	89-740	ITC and ThT-based label-free fluorescence assay	SCZ, SDM	This work
SQX-3	SDM, SQX, and SMZ	42 nt	320	SGI-based label-free fluorescence assay	SQX	This work

291 SMD: Sulfametoxydiazine; SCP: sulfachloropyridazine; MBs: magnetic beads; GO: graphene oxide; BHQ-

292 cDNA: Black Hole Quencher 1-labeled complementary strand

Conclusions

In summary, we used a mixture of three SAs to select aptamers and obtained three kinds of aptamers with different specificities. One of them showed a similar binding to all tested SAs with K_d ranging from 0.22 μ M to 0.63 μ M. For the other two aptamers, one is specific for SQX, and the other is specific for SDM and SCZ. The proposed label-free aptasensor was succeeded in applying to practical samples detection, with the LODs ranging from 0.14 μ M to 0.71 μ M in the lake water sample for the six SAs. The multi-targets SELEX strategy provides a shortcut for the generation of broad-spectrum aptamers for the detection of a family of antibiotics. Meanwhile, the proposed SGI-based label-free fluorescent aptasensors can work in lake water, and these aptamers are likely to be used in other sensor platforms for the detection of SA antibiotics.

Acknowledgements

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

DNA sequences used for aptamer selection and assays; the top 6 sequences from each of the sequenced library; the K_d of selected aptamers by using ThT-based assay and SGI-based assay; sequences used as aptasensors in previous studies; the ThT-based binding assays of SDM-3 and SQX-3; the optimal pH value optimization results of MIX-1 aptamer; the alignment results.

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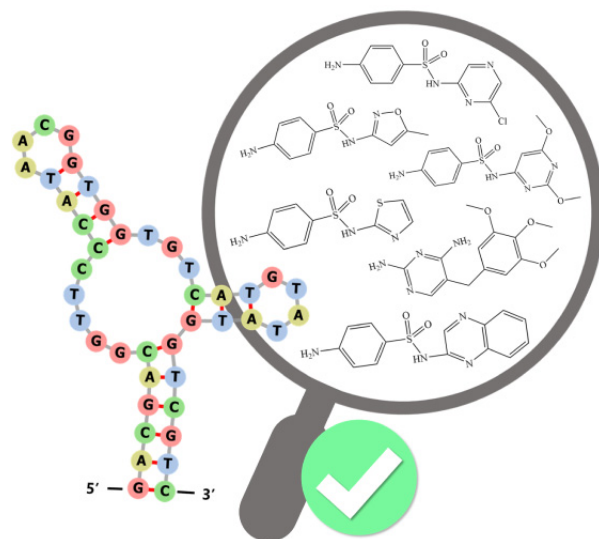
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